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Responsiveness of honey bee (*Apis mellifera* L.) corpora allata to allatoregulatory peptides from four insect species

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Abstract

Five neuropeptides with known allatotropic or allatostatic activity in other insect species were examined for their effects on honey bee corpora allata. Using an in vitro radiochemical assay, we assessed the ability of these peptides to affect the biosynthesis of juvenile hormone III and its immediate precursor methyl farnesoate, as well as their effects on the conversion of methyl farnesoate into juvenile hormone. None of the allatostatins tested affected JH biosynthesis during the last larval instar of honey bee workers. *Manduca sexta* allatotropin, however, stimulated JH biosynthesis in a stage-specific and dose-dependent manner. Analysis of intraglandular contents of juvenile hormone and its precursor revealed that the allatotropin significantly increased JH precursor but did not overcome the stage-specific block in the terminal step of JH biosynthesis that is typical for early fifth-instar worker larvae. Studies also indicated that the allatotropic effect was reversible at the level of methyl farnesoate production.

Keywords: JH biosynthesis; Allatotropin; Allatostatin; Caste development; *Apis mellifera*

1. Introduction

In honey bees (*Apis mellifera* L.), a fertilized egg can become either a worker or a queen and juvenile hormone (JH) is considered a prime inducer of queen development (Nijhout and Wheeler, 1982). Under normal circumstances, at the beginning of the last larval instar, corpora allata (CA) from honey bee larvae destined to become workers synthesize considerably less JH than CA from larvae destined to become queens. This is due to (1) a limited production of JH precursors and (2) a caste- and stage-specific block of the terminal step in JH biosynthesis (Rachinsky and Hartfelder 1990, 1991). Thus, both caste- and stage-specific differences in the regulation of JH biosynthesis seem to be responsible for the observed differences in JH production.

Since the first identification of peptides that regulate CA activity (Kataoka et al., 1989; Woodhead et al., 1989), a large number of peptides with allatoregulatory properties has been isolated from brains of insect species from different orders. Most of these neuropeptides are inhibitory (Stay et al., 1994; Bendena et al., 1997) and only one allatotropin has been isolated to date (Kataoka et al., 1989). The search for peptides that regulate JH biosynthesis in the honey bee has proven more difficult. While evidence exists for the presence of such peptides (Rachinsky, 1996) no identification has been forthcoming. In an effort to elucidate the role that allatoregulatory peptides may play in governing JH biosynthesis in the honey bee, we have examined the effect of five peptides from four insect species on larval honey bee corpora allata.

2. Materials and methods

2.1. Bees

Honey bee larvae were collected from queenright *Apis mellifera* colonies maintained at an apiary in Beltsville, MD. For in vitro experiments, fifth instar worker larvae were selected that were either (i) at the very beginning

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of the feeding stage (L5F1; 30–60 mg body weight), (ii) in the middle of the feeding stage (L5F2; 61–100 mg body weight) or (iii) at the beginning of the prepupal phase (PP1). PP1 larvae could be classified by measuring the tibio-tarsal length of the developing hind legs (1.40–1.99 mm). Feeding stage larvae and prepupae differ markedly with regard to CA activity (Rachinsky and Hartfelder, 1990). In stage PP1, CA activity is usually higher than during the feeding stages. Prepupae and feeding stage larvae were chosen because they have also been shown to exhibit stage-specific differences in the regulation of JH production. Stimulation of prepupal worker CA with farnesoic acid (FA) results in a large increase of JH production, whereas stimulation of feeding stage worker CA results primarily in a buildup of methyl farnesoate (MF), a JH precursor (Rachinsky and Hartfelder, 1991).

2.2. Radiochemical assay

Dissection of CA was carried out in a saline specifically designed for honey bee larvae (Rachinsky and Hartfelder, 1998). Excised CA were immediately transferred to 50 μl of methionine-free larval honey bee medium (Rachinsky and Hartfelder, 1998) and preincubated at room temperature for 10–20 min prior to the radiochemical in vitro assay (Pratt and Tobe, 1974; Tobe and Pratt, 1974). After preincubation in unlabeled medium, each pair of glands was transferred to 25 μl medium supplemented with 50 μM L-[^{14}C-methyl-] methionine (sp. act. 57.9 mCi/mmol; New England Nuclear, Boston, MA). This concentration of methionine was shown to yield optimal release rates of JH, when tested over a range between 20 and 200 μM methionine (Rachinsky and Hartfelder, 1998). Glands were usually (except for the assays to determine reversibility of peptide effects; see below) incubated for 2 h at 34.0°C with gentle shaking. Both preincubations and incubations were carried out in a humid chamber, in U-shaped acrylic plastic wells (LINBRO® 96-well tissue culture plates; 0.2 ml well capacity; ICN Biomedicals, Aurora, OH). At the end of the incubation period, CA and culture medium were separated. As a measure of JH biosynthesis, the rates of JH released into the medium were determined using a rapid partition radiochemical assay (Feyereisen and Tobe, 1981). The medium was extracted with 300 μl isooctane in 6×50 mm borosilicate tubes. An aliquot taken from the isooctane hyperphase was assayed by liquid scintillation spectrometry to determine JH release. The CA were extracted separately with chloroform/methanol according to the method described by Feyereisen (1985). The in vitro labeled products extracted from the glands were separated by thin-layer chromatography (TLC) using a hexane/ethyl acetate (3:1) solvent system. The separate analysis of glands and medium permitted the determination of JH release rates, as well as the quantification of intraglandular amounts of JH and its precursor methyl farnesoate.

2.3. Peptide source and treatments

Synthetic *Manduca sexta* allatotropin (Mas-AT; Kataoka et al., 1989) was obtained from Sigma (St. Louis, MO). The four synthetic allatostatins (designations according to Bendena et al., 1997) used in this study were: (1) Dip-AST 7, originally isolated from *Diploptera punctata* (Woodhead et al., 1989), (2) Cav-AST5 (*Calliphora vomitoria*; Duve et al., 1993), (3) Mas-AST (*M. sexta*; Kramer et al., 1991), and (4) a putative allatostatic peptide that was originally isolated from *Drosophila melanogaster*. This peptide has an amino acid sequence similar to the sequence of Mas-AST, the only difference being the substitution of a tyrosine for phenylalanine in position 4 (Tobe et al., 1995). All four allatostatins were generously provided by Stephen S. Tobe, University of Toronto.

Aequeous stock solutions of all peptides were prepared and aliquots sufficient for one experiment were stored at −20°C. Peptide solutions were diluted to final concentrations of 10^{-4} to 10^{-9} M with labeled incubation medium immediately prior to initiating the radiochemical in vitro assay. For every peptide concentration, 4–15 individual incubations each with one pair CA were carried out. The glands were incubated for 2 h in either labeled control medium (no peptide) or labeled medium containing peptide, then medium and glands were separated and extracted as described above. The stimulatory or inhibitory activity of the peptides was assessed by comparing JH release rates in control medium with those in medium containing peptide. The analysis of intraglandular JH and MF content was only carried out when JH release rates indicated that a particular peptide may be effective as a regulator of CA activity. JH release rates were calculated as pmol/pair CA h^{-1}, intraglandular contents of JH or MF were expressed as pmol/pair CA.

2.4. Tests on reversibility of peptide effects

Reversibility tests were only carried out with the *M. sexta* allatotropin. Three groups of CA, each consisting of 10–12 individual pairs of CA from the worker stage L5F1 were subjected to different incubation modes, consisting of an initial incubation and a subsequent incubation. In the first group (A), glands were incubated for two consecutive 2-h periods in control medium; in the second group (B), glands were incubated for 2 h in medium with 10^{-4} M Mas-AT followed by a 2-h incubation in control medium; and in the third group (C), glands were incubated for two consecutive 2-h periods in medium with Mas-AT. All incubations were in 25 μl of medium and glands were rinsed in control medium

between successive incubations. After the second incubation period, the CA and incubation medium from all three groups were collected for separate extraction, along with the media from the initial incubation.

2.5. Statistics

Mean rates (±SEM) of JH release were calculated by measuring 4–15 individual pairs of CA per concentration, or by determining JH and MF contents in two to six individual extractions. Statistical analyses included analysis of variance (ANOVA) followed by Tukey–Kramer Multiple Comparisons Test. Differences were considered significant when the P value was <0.05.

3. Results

3.1. Responsiveness of honey bee CA to allatostatic peptides

The four synthetic allatostatins utilized in this study did not have a significant effect on JH release by either CA from feeding stage workers (L5F1) or from worker prepupae (PP1). In several series of experiments, JH release in L5F1 controls varied from 0.648±0.078 to 0.774±0.062 pmol/pair CA h^{-1}. In PP1 controls, JH release varied from 1.149±0.128 to 1.389±0.053 pmol/pair CA h^{-1}. JH release rates from glands treated with the four allatostatins (Dip-AST7, Cav-AST5, Mas-AST, *D. melanogaster* allatostatin) over a wide range of concentrations (10^{-9} to 10^{-4} M) did not differ significantly from these respective control values.

3.2. Responsiveness of honey bee CA to M. sexta allatotropin (Mas-AT)

Mas-AT exhibited a stage-specific effect on JH release, stimulating JH release from feeding stage (L5F1) larval CA, but not from prepupal CA (Fig. 1).

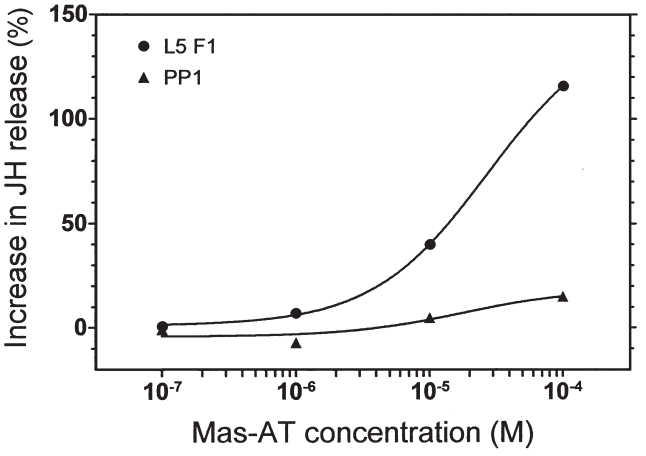

Fig. 1. In vitro effect of Mas-AT on JH release by honey bee corpora allata. JH release was calculated as the percent increase over respective control values and represented 4–15 replicates per concentration. L5F1, feeding stage worker larvae (30–60 mg); PP1, early prepupae.

In the feeding stage workers, the effect of Mas-AT was dose dependent. A 10^{-4} M Mas-AT concentration caused a highly significant ($P<0.001$) increase in JH release in L5F1 (1.600±0.119 pmol/pair CA h^{-1} versus 0.741±0.086 pmol/pair CA h^{-1} in untreated control glands). In prepupal CA exposed to 10^{-4} M Mas-AT, JH release (1.730+0.052 pmol/pair CA h^{-1}) was not significantly different from the respective control value (1.504±0.062 pmol/pair CA h^{-1}).

A more detailed examination of the effects of Mas-AT on honey bee CA was conducted by comparing JH release rates with the intraglandular contents of JH and its precursor MF. An additional developmental stage (L5F2) was also included in this examination. In control incubations, while JH release rates generally increase during the last larval instar, the only significant increase occurred in PP1 prepupae compared with the L5F1 stage (Fig. 2a). Mas-AT treatment (10^{-5} or 10^{-4} M) significantly elevated JH release rates from CA of both feeding

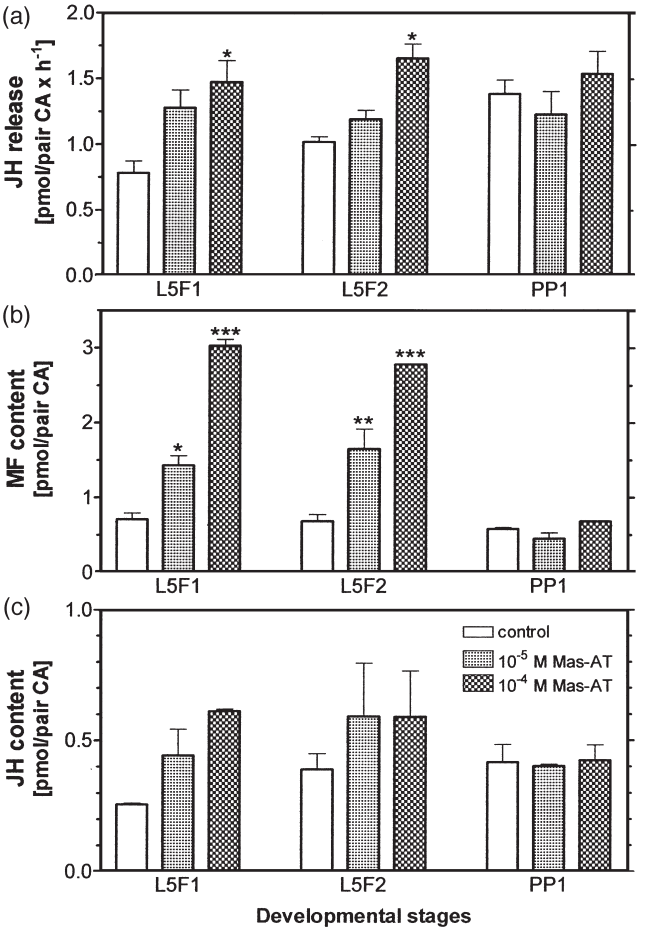

Fig. 2. Effects of Mas-AT on (a) JH release rates and intraglandular contents of (b) methyl farnesoate (MF) and (c) JH in larval and prepupal honey bees. Bars represent the means±SEM of two to six replicates. Asterisks denote significant differences from respective control values (ANOVA; $*P\leq0.05$; $**P\leq0.01$; $***P\leq0.001$). L5F1, feeding stage worker larvae (30–60 mg); L5F2, feeding stage worker larvae (61–100 mg); PP1, early prepupae.

stage larvae over their respective controls, and to the same levels that were measured in prepupae (Fig. 2a). In PP1 prepupae, no Mas-AT-dependent increase in JH release was observed over controls.

Mas-AT caused a significant ($P<0.01$ at 10^{-4} M; $P<0.001$ at 10^{-5} M Mas-AT) and dose-dependent increase in the production of the JH precursor MF in both L5F1 and L5F2 larvae (Fig. 2b). Maximally stimulated CA contained MF in the amounts of 3.027±0.086 (L5F1) and 2.780±0.006 (L5F2) pmol MF/pair CA. This reflects a 4.28-fold and 4.07-fold increase, respectively, above control levels of MF. In contrast, in prepupal CA, MF content was not affected by Mas-AT treatment.

The JH content of L5F1, L5F2 or PP1 glands was not affected by the Mas-AT treatment (Fig. 2c). In L5F1 glands, Mas-AT seemed to cause a dose-dependent increase in JH content at 10^{-5} M, but due to the high variation among individual measurements at this dose, these changes proved not to be significant ($P=0.05$).

3.3. Reversibility of Mas-AT effects on JH production

To determine if the effects of Mas-AT on JH production in feeding-stage worker CA were reversible, three groups of glands were subjected to different incubation modes consisting of two consecutive 2-h incubations as described in Section 2: (A) control–control media, (B) Mas-AT–control media, (C) Mas-AT–Mas-AT media.

The effect of Mas-AT on JH release did not appear to be reversible (Fig. 3). In group B, JH release in the second 2-h incubation (control medium) was not significantly different from the first 2-h incubation during which the CA were incubated in medium with Mas-AT; it was also not significantly different from JH release rates in glands that had been exposed to Mas-AT for two successive incubation periods (group C). In both groups B and C, and during both 2-h intervals, JH release rates were significantly different ($P<0.01$) from those in the respective intervals in control group A. Although we observed Mas-AT-related changes in JH release rates during both incubation periods in groups B and C, we could not detect any significant changes in intraglandular JH content (data not shown).

However, the Mas-AT effect seemed to be reversible on the level of MF production (Fig. 4). Glands that had first been incubated in Mas-AT medium and then for 2 h in control medium (group B) had a significantly lower MF content than glands that had been incubated in Mas-AT medium during both 2-h intervals (group C). Thus, under persistent stimulation with Mas-AT for 4 h, more MF was accumulated. The MF content of the CA in group B was higher than the MF content in group A, although this difference was not significant ($P<0.06$).

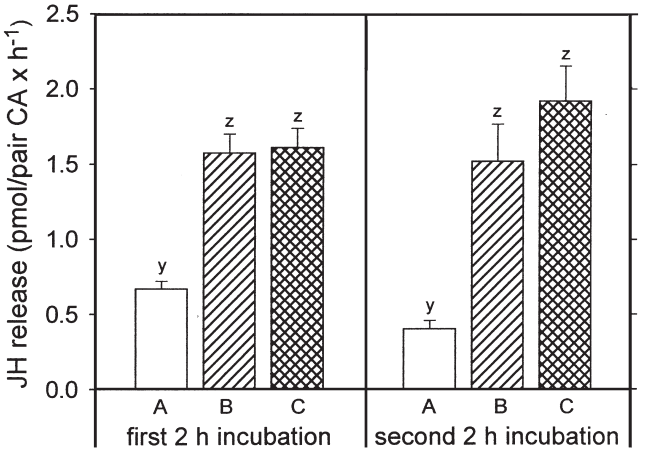

Fig. 3. Reversibility of Mas-AT effects on JH release from corpora allata of feeding stage worker lavae (L5F1). Glands were incubated for two successive 2-h periods in: (A) control medium followed by control medium; (B) Mas-AT medium followed by control medium; (C) Mas-AT medium followed by Mas-AT medium. Medium containing Mas-AT was at 10^{-4} M. Bars represent the means±SEM of 10–12 replicates. Letters (y,z) indicate significant difference in JH release (ANOVA; $P\leq0.01$).

4. Discussion

From the large number of known insect neuropeptides with allatostatic properties (Bendena et al., 1997), we examined four allatostatins that had been isolated from four different species representing three orders: Dip-AST 7 (*D. punctata;* Woodhead et al., 1989), Cav-AST5 (*C. vomitoria*; Duve et al., 1993), Mas-AST (*M. sexta*; Kramer et al., 1991), and a *D. melanogaster* peptide

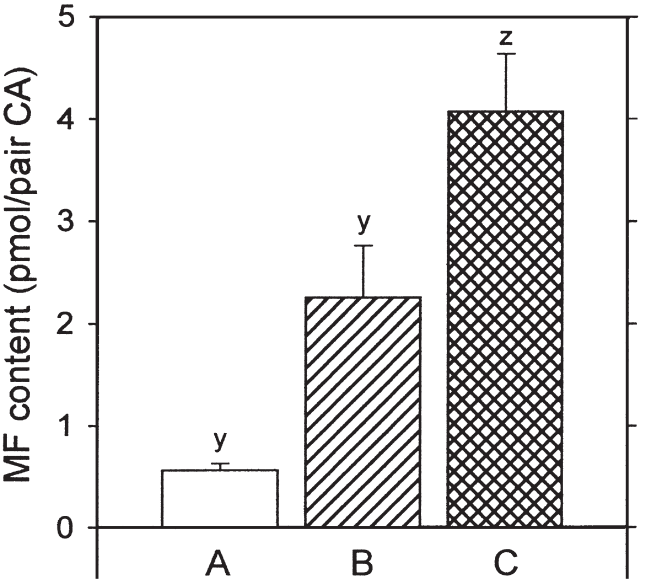

Fig. 4. Reversibility of Mas-AT effects on the intraglandular methyl farnesoate (MF) content from feeding stage worker larvae (L5F1). Incubation conditions are as in Fig. 3. Glands were extracted and analyzed for MF at the end of the 4-h incubation period. Letters (y,z) indicate significant difference in MF content (ANOVA; $P\leq0.05$).

(Tobe et al., 1995). These four peptides differed in structural characteristics. Dip-AST7 and Cav-AST5 belong to a large peptide family whose members are amidated and show sequence homologies at the C-terminal (Bendena et al., 1997). The sequences of Mas-AST and the *D. melanogaster* peptide are the same except for one amino acid (Tobe et al., 1995), but have no similarity with the sequences of the members of the former peptide family. Although *D. punctata* allatostatin-like immunoreactive material appears to be present in the brain and suboesophageal ganglion of honey bees (Stay et al., 1994), Dip-AST7 and Cav-AST5 did not inhibit JH biosynthesis by honey bee CA. The second type of allatostatic peptides also failed to have an effect on larval honey bee CA. This result is not completely surprising, since these allatostatins have previously shown fairly narrow specific activity. The Dip-ASTs, Cav-ASTs and other members of the same peptide family have shown allatostatic activity only in species of the orders Dictyoptera and Orthoptera, but not in the fly *C. vomitoria* in which such peptides also occur but may have different functions (Stay et al., 1994; Bendena et al., 1997). Mas-AST was only active in *M. sexta* and other lepidopteran species, but not in members of other insect orders (Kramer et al., 1991). Given this narrow specificity, the existence of endogenous honey bee allatostatins still remains a distinct possibility.

M. sexta allatotropin is the only insect neuropeptide with allatotropic properties identified to date. According to Kataoka et al. (1989), the stimulatory action of Mas-AT on JH biosynthesis seems to be restricted to species of the order Lepidoptera; Mas-AT did not affect CA activity in other insects like *Tenebrio molitor, Periplaneta americana* or *Schistocerca nitens*. Within lepidopteran species, its activity also seems to be further limited to adult CA.

The Mas-AT enhancement of the activity of larval honey bee CA is the first report of stimulatory effects of Mas-AT on CA of a non-lepidopteran species. The effect of Mas-AT on larval honey bee CA was stage-specific. CA from feeding stage worker larvae of the last larval instar were stimulated by treatment with Mas-AT, while the CA from worker prepupae were unaffected. This result has implications regarding JH involvement in the regulation of caste development in honey bees. Under unstimulated conditions, the highest JH biosynthesis rates occur in feeding stage queen larvae (Rachinsky and Hartfelder, 1990). Such rates in larval JH production are most likely caused by stimulatory signals from the brain, perhaps neuropeptides, and evidence for the presence of allatotropic material in bee brains has recently been presented (Rachinsky, 1996).

The effect of Mas-AT on JH production in feeding stage worker CA was reversible at the level of MF production, but not with regard to JH release rates. This reversal effect upon intraglandular precursor levels is in agreement with previous observations of the JH biosynthetic pathway. We know from earlier experiments with farnesoic acid, a precursor of MF, that the terminal epoxidation step in JH biosynthesis is caste- and stage-specifically blocked in feeding stage worker larvae (Rachinsky and Hartfelder, 1991). Stimulation of CA with farnesoic acid results in an enormous accumulation of MF in L5F1 CA. In the present study, Mas-AT also caused a considerable accumulation of MF in feeding stage worker CA (L5F1 and L5F2). This demonstrates that Mas-AT activates the production of JH precursor, presumably by stimulating early steps in JH biosynthesis, but does not overcome the limitation in epoxidation activity which is typical for feeding stage worker larvae. Despite higher basal rates of JH release in L5F2 compared with L5F1, the allatotropin-stimulated JH release rates were about the same for both stages. JH release rates were also at about the same level when L5F1 CA were stimulated with either active fractions from prepupal brain extracts (Rachinsky, 1996) or with biogenic amines (Rachinsky, 1994). Taken together, this may indicate that hourly JH release rates of about 1.6 pmol/pair CA may indeed reflect the maximum capacity of the epoxidation step in JH production in feeding stage worker. Accordingly, in the case of Mas-AT stimulation, only a fraction of the precursor produced was converted into JH and released into the medium; the remaining fraction accumulated within the CA. The limitation in epoxidation activity may also explain why we did not observe a return of JH release to control levels after transferring CA from Mas-AT medium into control medium. Analysis of the MF content of the glands revealed that MF levels were lower in glands incubated under such conditions, than in glands that were incubated in Mas-AT medium during the entire 4-h incubation period, and were also lower than in glands that were incubated for only 2 h in Mas-AT medium. It appears that MF was only produced and accumulated as long as the Mas-AT stimulus was present. Thus, the stimulatory effect of Mas-AT on MF production was reversed when glands were transferred into control medium. However, JH release rates may stay elevated for several hours due to ongoing conversion into JH of previously accumulated MF.

Larval honey bee CA responded to rather high concentrations of Mas-AT (10^{-5} or 10^{-4} M), relative to *M. sexta* CA, which were stimulated at concentrations in the nanomolar range (Kataoka et al., 1989). Assuming that Mas-AT acts on CA activity via receptor-mediated processes, honey bee CA have receptors for Mas-AT, albeit with low affinity. This suggests that honey bee-specific peptides with characteristics similar to Mas-AT may exist. Despite the potential pitfalls associated with interpreting experiments in which the regulatory hormones of one species are tested in another species, the results obtained with Mas-AT and honey bees may facilitate the

isolation of honey bee-specific allatotropins. Both stage-specific differences in the responsiveness of honey bee CA to Mas-AT, as well as the reversibility of Mas-AT effects on JH precursor production indicate a specific stimulation of JH biosynthesis rather than a non-specific effect on overall CA metabolism.

With regard to JH biosynthesis regulation by the central nervous system (CNS), or by factors originating in the CNS, insect species can be grouped into two basically different types of regulation (Nijhout, 1994). In some species, like *D. punctata, Leucophaea maderae* and *Rhodnius prolixus*, the CA appear to be mainly under inhibitory control via nerves and/or neurosecretions. In other insects, like in *M. sexta* larvae or *Locusta migratoria* adults, JH biosynthesis seems to require stimulatory signals. Honey bee larvae apparently belong to this second type of JH biosynthesis regulation and, thus, it is not surprising that their CA seem to lack any sensitivity to allatostatic peptides, but respond to Mas-AT. In larval honey bee CA, JH biosynthesis rates may be generally low and dependent on an exact timing of stimulatory signals in developmental stages during which high JH titers are required for caste-specific development, e.g. during the first half of the last larval instar of queen larvae. Our results with Mas-AT suggest that there may be structurally related peptides present in honey bee larvae, regulating caste- and stage-specific production of JH. We are currently investigating the stage-specific pattern of Mas-AT action on honey bee CA.

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